Muchmore, D. B., Little, S. A., & de Haën, C. (1981) *J. Biol. Chem. 256*, 365–372.
Mukherjee, S. P., & Lynn, W. S. (1977) *Arch. Biochem. Biophys. 184*, 69–76.
Mukherjee, S. P., Lane, R. H., & Lynn, W. S. (1978) *Biochem. Pharmacol. 27*, 2589–2594.
Prilop, B. (1975) Thesis, Medizinische Hochschule Hannover, West Germany.
Robison, G. A., Butcher, R. W., & Sutherland, E. W. (1971) *Cyclic AMP*, p 295, Academic Press, New York.
Rodbell, M. (1964) *J. Biol. Chem. 239*, 375–380.
Schimmel, R. J., Buhlinger, C. A., & Serio, R. (1980) *J. Lipid Res. 21*, 250–256.
Sengupta, K., Long, K. J., & Allen, D. D. (1981) *J. Pharmacol. Exp. Ther. 218*, 128–133.
Stock, K., & Prilop, M. (1974) *Naunyn-Schmiedeberg's Arch. Pharmacol. 282*, 15–31.
Wieser, P. B., & Fain, J. N. (1975) *Endocrinology (Philadelphia) 96*, 1221–1225.
Wieser, P. B., & Pendleton, T. S. (1979) *Biochem. Pharmacol. 28*, 693–694.
Wong, E. H.-A., & Loten, D. G. (1981) *Eur. J. Biochem. 115*, 17–22.

Biosynthesis, Nonenzymatic Synthesis, and Purification of the Intermediate in Synthesis of Sepiapterin in *Drosophila*†

Dale Dorsett,‡ John M. Flanagan,§ and K. Bruce Jacobson*

ABSTRACT: The enzymatic conversion of the D-*erythro*-dihydroneopterin triphosphate [H_2-neopterin-$(P)_3$] to sepiapterin occurs via a nonphosphorylated intermediate as shown by others. We have developed a high-performance liquid chromatography assay for this intermediate and have found that the intermediate (X) and two related compounds (X1 and X2) can be formed nonenzymatically under certain conditions from H_2-neopterin-$(P)_3$. The reaction is catalyzed by tris(hydroxymethyl)aminomethane, dependent upon H_2-neopterin-$(P)_3$ concentration, significant at temperatures greater than 80 °C, and maximal between pH 8.5 and 9.5 (as determined at 25 °C). All three compounds were purified, and it was found that both X and X1 can serve as substrates for the enzymatic, NADPH-dependent synthesis of sepiapterin. From the kinetics of formation from H_2-neopterin-$(P)_3$ and the similarity of the ultraviolet spectra, it is clear that X, X1, and X2 are closely related compounds. None of the three compounds is reduced by $NaBH_4$; only X1 is sensitive to periodate oxidation. All three can be oxidized with iodine to give rise to highly fluorescent compounds that in turn can be reduced by $NaBH_4$ to give rise to the respective parent compounds. These latter observations indicate that X, X1, and X2 are dihydropterins. These results are discussed relative to the proposed structures for enzymatically produced X. The methods described for the nonenzymatic synthesis of X and its purification should allow preparation of large amounts of X for future study.

The enzymatic conversion of the D-*erythro*-dihydroneopterin triphosphate [H_2-neopterin-$(P)_3$][1] to 7,8-dihydro-6-lactoylpterin (sepiapterin) occurs in the presence of Mg^{2+} and NADPH. It has been demonstrated that sepiapterin synthase from *Drosophila melanogaster* (Dorsett et al., 1979; Krivi & Brown, 1979) and chicken kidney (Tanaka et al., 1981) consists of two enzymes, the first requiring Mg^{2+} as a cofactor and the second requiring NADPH. The separation of these two activities from *Drosophila* (Krivi & Brown, 1979) and from chicken kidney (Tanaka et al., 1981) has been reported. It has also been proposed that the product of the Mg^{2+}-dependent first enzyme is a precursor of the *Drosophila* red eye pigments, the "drosopterins", as well as of sepiapterin (Dorsett et al., 1979; Dorsett & Jacobson, 1982).

Following an earlier report (Dorsett et al., 1980), we describe here an assay for the sepiapterin synthase intermediate (the product of the Mg^{2+}-dependent first enzyme) using high-performance liquid chromatography (HPLC). A nonenzymatic method for the conversion of H_2-neopterin-$(P)_3$ to the intermediate (X) and related compounds (X1 and X2) is described, as well as the purification and partial characterization of these compounds. The implications of these studies with regard to the structure of the intermediate and the mechanism of sepiapterin synthesis are discussed.

Materials and Methods

Chromatography columns were obtained from Waters Associates (C_{18} μBondapak) and Du Pont (C8 Zorbax). Thin-layer cellulose sheets (no. 13255) were from Eastman Kodak Co.; methanol was purchased from Burdick and

† From the University of Tennessee—Oak Ridge Graduate School of Biomedical Sciences and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. *Received March 15, 1982.* This research was sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract W-7405-eng-26 with the Union Carbide Corporation.

* Address correspondence to this author at the Biology Division, Oak Ridge National Laboratory.

‡ Predoctoral fellow supported by Genetics Training Grant GM7438 from the National Institute of General Medical Sciences. This research was done in partial fulfillment of the Ph.D. thesis requirements of the University of Tennessee.

§ Graduate assistant supported by the University of Tennessee.

[1] Abbreviations: GTP, guanosine 5′-triphosphate; H_2-neopterin-$(P)_3$, 3′-triphosphoester of 7,8-dihydro-6-(D-*erythro*-1,2,3-trihydroxypropyl)-pterin; sepiapterin, 7,8-dihydro-6-lactoylpterin; xanthopterin, 6-hydroxypterin; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; CE, crude enzyme extract; PFE, pteridine-free extract.

0006-2960/82/0421-3892$01.25/0

Jackson, Inc.; Pipes was from Calbiochem, Sephadex G-25 was from Pharmacia, and DEAE-cellulose was DE-23 from Whatman. The sources of various pteridines was described previously (Wilson & Jacobson, 1977). Sepiapterin was prepared from *Drosophila* heads as described (Dorsett et al., 1979). From Amersham we obtained [U-^{14}C]GTP (20 mCi/mmol) and from New England Nuclear [8,5′-^{3}H]GTP (37.6 Ci/mmol).

Drosophila melanogaster. Oregon-R and *pr*^{bw}*cn* flies were reared at 25 ± 1 °C on medium prepared as described by Lewis (1960). Flies were collected daily so that all were 1-day-old or less. After collection, the flies were quickly frozen in liquid nitrogen; the heads were obtained as previously described (Wilson & Jacobson, 1977) and stored in vials under liquid nitrogen for later use.

Crude Enzyme Extract (CE). Heads (1 g) were homogenized in 5 mL of 50 mM 1,4-piperazinediethanesulfonic acid (Pipes, pH 7.0) with a ground-glass homogenizer (Kontes). The resulting homogenate was centrifuged at 15000*g* for 10 min, and the supernatant was centrifuged at 100000*g* for 60 min. The protein, precipitating between 40 and 60% saturated $(NH_4)_2SO_4$, was collected at 15000*g* for 30 min, redissolved in 0.2 mL of 50 mM Pipes (pH 7.0), and filtered through 20 mL of Sephadex G-25 (medium) by using the centrifuge procedure of Neal & Florini (1973). The preparation contained 20 mg of protein/mL and was stored at −20 °C.

Pteridine-Free Extract (PFE). PFE was prepared as previously described (Dorsett et al., 1979), except that the pellet resulting from 60% saturated $(NH_4)_2SO_4$ was redissolved in 1 mL of 50 mM Pipes (pH 7.0) containing 10% glycerol and desalted by filtration through 20 mL of Sephadex G-25 (medium) using the centrifuge procedure. The extract contained 15 mg of protein/mL and was stored at −20 °C.

H_2-neopterin-(P)$_3$. H_2-neopterin-$(P)_3$ was prepared by using pure *Escherichia coli* GTP cyclohydrolase I that was prepared as described by Yim & Brown (1976). The reaction contained 1.67 mM GTP, 0.11 M Tris-Cl (pH 8.5), 0.11 M NaCl, 0.011 M EDTA (pH 7.5), and enough enzyme to achieve at least a 90% conversion in 90 min at 42 °C as determined by the release of the 8-^{14}C of GTP as formate. The completed GTP cyclohydrolase reaction mixture was used directly as the source of H_2-neopterin-$(P)_3$. All H_2-neopterin-$(P)_3$ concentrations were calculated assuming 100% conversion. H_2-neopterin-$(P)_3$ was treated, when appropriate, with bacterial alkaline phosphatase as previously described (Dorsett et al., 1979).

Separation of H_2-neopterin-$(P)_3$ from the other ingredients of the cyclohydrolose reaction was achieved by chromatography in a DEAE-cellulose column (0.6 × 30 cm) at 4 °C in the dark using a 500-mL linear gradient from 0.05 M to 0.2 M ammonium bicarbonate and collecting 7.5-mL fractions. The peak tubes, observed by measuring A_{330}, A_{260}, and fluorescence (excite 350, emit 450 nm; Aminco Bowman spectrofluorometer was standarized by using 10 μM anthranilic acid) were pooled, lyophilized, and redissolved in 500 μL of water. The concentration of H_2-neopterin-$(P)_3$ was estimated by using a molar extinction coefficient of 6.2 × 10^3 at 330 nm (Tsusue & Akino, 1965). Thin-layer chromatography of these pools was performed by using a developer consisting of 57 mL of isobutyric acid, 3 mL of concentrated NH_4OH, 39 mL of H_2O (Circular OR-10, 1956), and a cellulose sheet (Eastman 13255).

Analysis for X, X1, X2, and Sepiapterin. A Waters liquid chromatograph with a 3.6 mm × 30 cm C_{18} or a 3.6 × 25 cm C_8 reverse-phase column was used to separate sepiapterin and X from the reactions since Fukushima & Nixon (1979a) had observed that other pteridines were well resolved on this column. The column was eluted at 2 mL/min with 10% methanol for the C_{18} column or 12% methanol for the C_8 column at a temperature of 35 °C. The pterins were quantitated by using a Schoeffel SF 770 UV monitor at 260 nm set to 0.1 aufs with a 1-mV recorder and a Schoeffel FS 970 fluorescence monitor (excitation at 360 nm, Corning 7-54 excitation filter, 418-nm cutoff emission filter) set to 0.1 μA (4-s time constant) with a 1-mV recorder. Determination of the molar concentration products in the peaks was accomplished by using [U-^{14}C]H_2-neopterin-$(P)_3$ as a substrate (specific activity 20.3 μCi/μmol). Sepiaterin concentration was calculated from the millimolar extinction coefficient of 10.4 at 420 nm (Matsubara et al., 1960).

Preparation of Purified X, X1, and X2. Aliquots (0.5–1.0 mL) of the completed GTP cyclohydrolase reaction contained 1.67 mM H_2-neopterin-(P_3), 1.67 mM formate, 0.11 M Tris-HCl (pH 8.5), 0.11 M NaCl, and 0.011 M EDTA, were heated at 100 °C for 40 min in plastic, capped tubes, and then were centrifuged. The X, X1, and X2 in the supernatant were separated by HPLC (as above, except that the column was eluted with 5% methanol to increase resolution), and fractions containing the individual species were lyophilized to dryness. The pterins were redissolved in approximately 100 μL of H_2O, and the purity was checked by HPLC. Occasionally the X1 fraction had to be rechromatographed due to significant impurities. Although this resulted in some loss of X1, the second purification was always sufficient. The compounds were stored at −20 °C and were stable for a few days.

Enzymatic Synthesis of X and Sepiapterin. The exact conditions (enzyme, substrate, and cofactors present, and reaction time) are shown for the individual experiments in Table I. When radioactive H_2-neopterin-$(P)_3$ was used as a substrate, fractions were collected in scintillation vials, and the radioactivity was determined in a scintillation counter after the addition of 10 volumes of 0.28% 2,5-bis[2-(5-*tert*-butylbenzoxalyl)]thiophene (Packard) and 33% Triton X-100 in toluene.

Nonenzymatic Synthesis of X, X1, and X2. The exact conditions for the experiments are given in the figure legends. Aliquots of the reactions were analyzed by HPLC after centrifugation (Brinkmann Eppendorf Centrifuge 3200).

Phosphate Analysis. The following solution was prepared in a total volume of 100 μL: 1 mM H_2-neopterin-$(P)_3$, 0.067 M Tris-HCl (pH 8.5), 0.067 M NaCl, 0.0067 M EDTA (pH 7.5), and up to 20 μL of either 0.25 M NaOH or 0.25 M HCl to achieve the desired pH. It was incubated at 100 °C for 10 min, cooled in ice, and analyzed for phosphate by the method of Sumner (1944). Seven hundred microliters of H_2O, 100 μL of 6.6% ammonium molybdate in 7.5 N H_2SO_4, and 100 μL of $FeSO_4$ solution (2.5 g of $FeSO_4 \cdot 7H_2O$ dissolved in 25 mL H_2O plus 0.5 mL of 7.5 N H_2SO_4) were added to each reaction. The solutions were mixed and incubated at room temperature for 15 min; the absorbance at 660 nm was determined. Phosphate concentrations were calculated from a standard curve.

Analysis for Side-Chain Loss. Reactions were run with a total volume of 50 μL containing the following components: 1 mM [3′-^{3}H]H_2-neopterin-$(P)_3$ (prepared from [8,5′-^{3}H]GTP, specific activity 2 mCi/mmol), 0.067 M Tris-HCl (pH 8.5), 0.067 M NaCl, 0.0067 M EDTA (pH 7.5), and up to 9 μL of 0.25 M NaOH to achieve various pHs. The solutions were incubated at 100 °C for 10 min, cooled in ice, and centrifuged. Next, H_2O (0.5 mL) was added to each reaction, and the amount of side chain released was determined by filtering

Table I: Biosynthesis of X and Sepiapterin[a]

Substrate, Enzyme, and Cofactor Requirement

enzyme (μL)	substrate (mM)	Mg^{2+}	NADPH	reaction time (min)	amt of product (nmol/mL) X	amt of product (nmol/mL) sepiapterin
Oregon-R-PFE (10)	Pase-H_2-neopterin-$(P)_3$ (0.143)	+	0	15	0	0
Oregon-R-PFE (10)	Pase-H_2-neopterin-$(P)_3$ (0.143)	+	+	15	0	0
ΔOregon-R-PFE (10)	H_2-neopterin-$(P)_3$ (0.143)	+	0	15	0	0
Oregon-R-PFE (10)	H_2-neopterin-$(P)_3$ (0.143)	0	0	15	0.37	0
Oregon-R-PFE (10)	H_2-neopterin-$(P)_3$ (0.143)	+	0	15	2.30	0
Oregon-R-PFE (10)	H_2-neopterin-$(P)_3$ (0.143)	+	+	15	1.30	5.43

Comparison of Oregon-R and $pr^{b}w^{cn}$ Activities

enzyme (μL)	substrate (mM)	Mg^{2+}	NADPH	reaction time (min)	amt of product (nmol/mL) X	amt of product (nmol/mL) sepiapterin	Oregon-R activity (%) X	Oregon-R activity (%) sepiapterin
Oregon-R-PFE (5)	H_2-neopterin-$(P)_3$ (0.286)	+	0	10	1.10	0	100.0	–
$pr^{b}w^{cn}$-PFE (5)	H_2-neopterin-$(P)_3$ (0.286)	+	0	10	0.23	0	21.2	–
Oregon-R-PFE (5)	H_2-neopterin-$(P)_3$ (0.286)	+	+	10	0.70	2.20	–	100.0
$pr^{b}w^{cn}$-PFE (5)	H_2-neopterin-$(P)_3$ (0.286)	+	+	10	0.13	0.43	–	19.7

Comparison of Radioactivity Incorporated from [U-^{14}C,3′-^{3}H]H_2-neopterin-$(P)_3$ into X and Sepiapterin

enzyme (μL)	substrate (mM)	Mg^{2+}	NADPH	reaction time (min)	dpm/mL X ^{3}H	dpm/mL X ^{14}C	dpm/mL sepiapterin ^{3}H	dpm/mL sepiapterin ^{14}C	^{3}H dpm/^{14}C dpm X	^{3}H dpm/^{14}C dpm sepiapterin
Oregon-R-PFE (5)	[U-^{14}C,3′-^{3}H]-H_2-neopterin-$(P)_3$[b] (1.56)	+	0	30	92 770	73 130	–	–	1.27	–
Oregon R-PFE (15)	[U-^{14}C,3′-^{3}H]-H_2-neopterin-$(P)_3$[b] (0.78)	+	+	30	–	–	429 700	317 970	–	1.35

Synthesis of Sepiapterin from ΔH_2-neopterin-$(P)_3$

enzyme (μL)	substrate (mM)	Mg^{2+}	NADPH	reaction time (min)	amt of product (nmol/mL) X	amt of product (nmol/mL) sepiapterin
Oregon-R-CE (15)	H_2-neopterin-$(P)_3$ (0.954)	0	+	60	–	1.97
Oregon-R-CE (15)	ΔH_2-neopterin-$(P)_3$ (0.954)	0	+	60	–	8.40
Oregon-R-CE (15)	ΔH_2-neopterin-$(P)_3$ (0.954)	0	0	60	–	1.30
ΔOregon R-CE (15)	ΔH_2-neopterin-$(P)_3$ (0.954)	0	+	60	–	1.00

Synthesis of Sepiapterin from Purified X and X1

enzyme (μL)	substrate (mM)	Mg^{2+}	NADPH	reaction time (min)	amt of product (nmol/mL) X	amt of product (nmol/mL) sepiapterin
Oregon-R-PFE (30)	X (0.013)	0	+	90	–	9.93
Oregon-R-PFE (30)	X (0.013)	0	0	90	–	0
ΔOregon-R-PFE (30)	X (0.013)	0	+	90	–	0
Oregon-R-PFE (30)	X1 (0.004)	0	+	90	–	2.70
Oregon-R-PFE (30)	X1 (0.004)	0	0	90	–	0
ΔOregon-R-PFE (30)	X1 (0.004)	0	+	90	–	0

[a] Reactions were performed in 70 μL that contained 50 mM Pipes (pH 7.5) and the other components as shown above (Mg^{2+} at 14.3 mM and NADPH at 2.5 mM). The solutions were incubated for the time shown at 42 °C and then at 100 °C for 5 min to stop the reaction. The coagulated protein was removed by centrifugation and 30-μL aliquots were analyzed for X and sepiapterin by HPLC. Abbreviations: Pase-H_2-neopterin-$(P)_3$, phosphatase-treated H_2-neopterin-$(P)_3$; (+), present in the reaction; (0), not present in the reaction; ΔOregon-R-PFE, Oregon-R-PFE heated at 100 °C for 5 min; (–), not measured; ΔH_2-neopterin-$(P)_3$, completed GTP cyclohydrolase reaction heated at 100 °C for 40 min; ΔOregon-R-CE, Oregon-R-CE heated at 100 °C for 5 min. Protein concentrations may be derived from the following: 30 μL of PFE equals 45 μg and 15 μL of CE equals 30 μg. [b] Specific activity = 2.03 μCi/μmol of ^{14}C and 37.69 μCi/μmol of ^{3}H.

through Pasteur pipet columns containing 0.5 × 1 cm acid-washed charcoal at the bottom and 0.5 × 1 cm DEAE-Sephadex A120 (equilibrated with 50 mM Pipes, pH 7.0) following the procedure of Yim et al. (1981). The side chain was eluted with two washes of 0.5 mL of H_2O, and the radioactivity present in the effluent was determined by scintillation counting.

Degradation of X at Various pH Values. The intermediate (X) was generated by incubating 1.67 mM H_2-neopterin-$(P)_3$ that was still dissolved in 0.11 M Tris-HCl (pH 8.5), 0.11 M NaCl, and 0.011 M EDTA (pH 7.5) at 100 °C for 10 min before cooling in ice. Aliquots (15 μL) of the heated H_2-neopterin-$(P)_3$ were adjusted to certain pH values by dilution to 25 μL with up to 5.5 μL of 0.25 M NaOH or 0.25 M HCl and H_2O. The reaction mixtures were incubated at 60 °C for 120 min and analyzed by HPLC.

Treatment of X, X1, and X2 with I_2, $NaBH_4$, and $NaIO_4$. Oxidations of X, X1, or X2 in a solution containing 0.1% I_2 and 0.2% KI were carried out in the dark at room temperature for up to 30 min; the excess I_2 was titrated out with a 1% ascorbic acid solution before analysis of the reaction by HPLC. So that the sensitivity of the iodine oxidation products to $NaBH_4$ could be tested, aliquots of the iodine reactions were adjusted to 0.05 M $NaBH_4$ with a solution containing 0.5 M $NaBH_4$ and 0.1 M NaOH and incubated in the dark at room temperature for various times up to 30 min before HPLC analysis. The unoxidized forms of X, X1, or X2 were treated with the same concentrations of $NaBH_4$ and NaOH. Per-

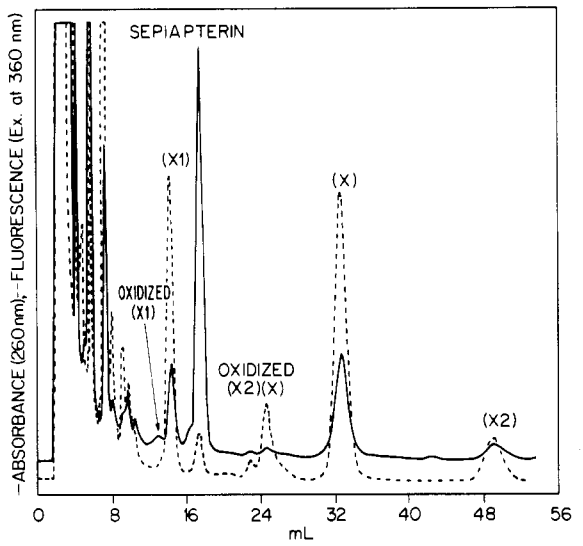

FIGURE 1: Separation of X, X1, X2, and sepiapterin by HPLC. The sample contained 1 nmol of sepiapterin plus the products obtained by heating 16 nmol of H_2-neopterin-$(P)_3$ in the presence of the GTP cyclohydrolase reaction ingredients at 100 °C for 40 min. The HPLC was performed on the C_{18} column with 10% methanol as described in the text, except that the fluorometer was set to the 1.0-μA range instead of the 0.1-μA range. Small amounts of the oxidized forms of X, X1, and X2 appear under these conditions.

iodate oxidations were carried out by adjusting solutions of X, X1, or X2 to 0.02 M $NaIO_4$ with a 0.2 M $NaIO_4$ (adjusted to pH 5 with 0.1 N HCl) solution. The reactions were incubated for various times up to 60 min at room temperature in the dark before analyzing aliquots by HPLC.

Results

The discovery of the nonenzymatic conversion of H_2-neopterin-$(P)_3$ to the sepiapterin synthase intermediate (X) occurred when the GTP cyclohydrolase reaction products were heated at 100 °C for 45 min. The identity of X was based on its chromatographic similarity to the intermediate produced by the partially purified enzyme. Indeed, on further study, two chromatographic peaks were found to be associated with X when it was produced nonenzymatically; these forms, X1 and X2, are not present in significant amounts with the enzymatically produced X. An HPLC chromatogram of nonenzymatically synthesized X, X1, and X2, along with added sepiapterin, is shown in Figure 1. Enzymatically synthesized X eluted in the same position as the nonenzymatically produced compound and demonstrated the same absorbance-to-fluorescence ratio.

Enzymatic Synthesis of X and Sepiapterin. Several lines of evidence support the hypothesis that the peak that elutes at 32 mL (Figure 1) is the same as the enzymatically synthesized X, the sepiapterin synthase intermediate. Treatment of H_2-neopterin-$(P)_3$ with bacterial alkaline phosphatase or heating of the enzyme extract at 100 °C for 5 min prior to use prevents the enzymatic synthesis of X (Table I). Enzymatic synthesis of X requires Mg^{2+}, whereas the recovery of X decreases when NADPH is added; this decrease is concomitant with an increase in the synthesis of sepiapterin (Table I). The *pr*^{bw}*cn* mutant, which contains only 20% of the sepiapterin synthase present in Oregon-R and is thought to be a lesion in the structural locus for the Mg^{2+}-dependent first enzyme of sepiapterin synthase (Yim et al., 1977; Krivi & Brown, 1979; Dorsett et al., 1979), has only 20% wild-type activity for X synthesis (Table I). Enzymatically produced X incorporates ^{14}C from [U-^{14}C]H_2-neopterin-$(P)_3$ and ^3H from [3′-^3H]H_2-neopterin-$(P)_3$ in the same ratio as sepiapterin (Table I).

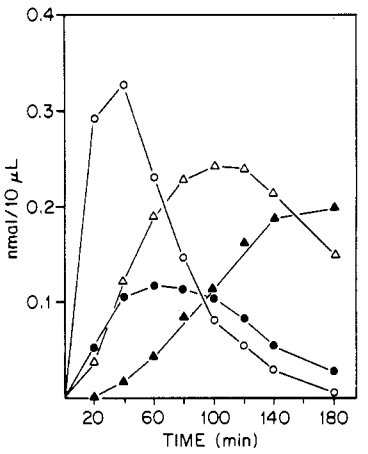

FIGURE 2: Time course of nonenzymatic synthesis of X (○), X1 (△), X2 (●), and oxidized X1 (▲). Tubes containing 1.67 mM H_2-neopterin-$(P)_3$, 0.11 M Tris-Cl (pH 8.5), 0.011 M EDTA (pH 7.5), and 0.11 M NaCl were incubated at 100 °C in the dark; aliquots were analyzed by HPLC at the times indicated. Amounts of oxidized X1 were calculated by assuming that its absorption coefficient at 260 nm is the same as that for X1 which is based on the radioactivity associated with the absorbance peak obtained from X, X1, and X2 derived from [U-^{14}C]H_2-neopterin-$(P)_3$.

More direct evidence that X is the intermediate and additional evidence that the X produced nonenzymatically is the same compound as X produced enzymatically were obtained by using nonenzymatically synthesized X as a substrate for the enzymatic synthesis of sepiapterin. the conditions for the synthesis of X from H_2-neopterin-$(P)_3$ nonenzymatically are examined in detail below. As mentioned above, when a GTP cyclohydrolase reaction that had gone to completion was heated at 100 °C, three related compounds (X, X1, and X2) were formed. It was found that such heated H_2-neopterin-$(P)_3$ solutions could support enzyme-dependent synthesis of sepiapterin in the presence of NADPH and the absence of Mg^{2+} (Table I). The lack of a Mg^{2+} requirement indicated that heating H_2-neopterin-$(P)_3$ could substitute for the Mg^{2+}-dependent enzymatic reaction of sepiapterin synthase. Furthermore, it was found that either X or X1 purified from the heated H_2-neopterin-$(P)_3$ solutions could serve as a substrate for the NADPH-dependent enzymatic synthesis of sepiapterin (Table I). Because the nonenzymatically produced X appears to be identical with the enzymatically produced intermediate by HPLC elution (and the relationship between absorbance and fluorescence), we propose that X is the actual sepiapterin synthase intermediate and that X1 is a derivative of X produced under the nonenzymatic reaction conditions. The third substance, X2, was produced in smaller amounts than X or X1, and upon storage at −20 °C it became converted to X1; (X2) was not tested as a substrate for sepiapterin synthase.

Nonenzymatic Synthesis of X, X1, and X2. The time course for the nonenzymatic synthesis of X, X1, and X2 from H_2-neopterin-$(P)_3$ is shown in Figure 2. First to be synthesized was X, which reached a maximum by 40 min and then fell off; X2 reached a maximum amount at 60 min and then declined; X1 reached a maximum at 100 min and then declined, with a concomitant increase in the amount of oxidized X1. Small amounts of oxidized X and oxidized X2 were also produced during the reaction (the identity of the oxidized forms was confirmed by iodine oxidations of purified X, X1, and X2 as described below). These results suggest the following reaction sequence: H_2-neopterin-$(P)_3$ is converted to X, which gives rise to X1 and X2, which in turn are oxidized.

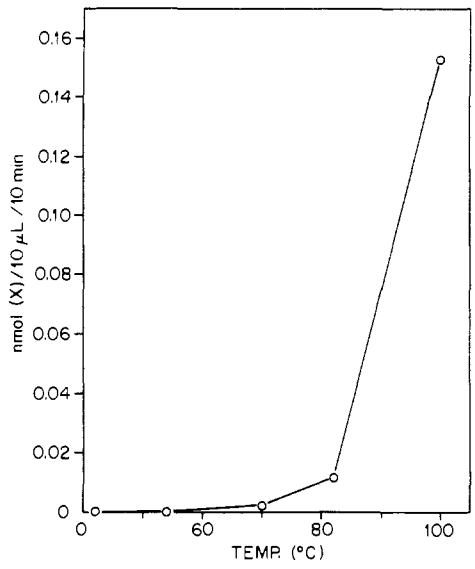

FIGURE 3: Rate of nonenzymatic synthesis of X as a function of temperature. Reactions contained 1.67 mM H_2-neopterin-$(P)_3$, 0.11 M Tris-HCl (pH 8.5), 0.011 M EDTA (pH 7.5), and 0.11 M NaCl. The reactions were incubated for 10 min at the temperatures indicated.

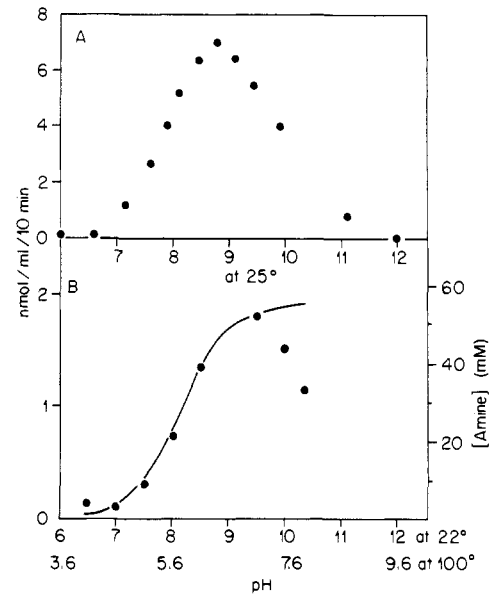

FIGURE 4: Rate of nonenzymatic synthesis of X as a function of pH. (A) Solutions (50 μL) contained 1 mM H_2-neopterin-$(P)_3$, 0.11 M Tris-HCl (pH 8.5), 0.067 M NaCl, 0.0067 M EDTA (pH 7.5), and sufficient amounts of NaOH or HCl to adjust the pH to the indicated values. The pH was measured with a glass electrode at 25 °C, and the reaction was performed at 100 °C for 10 min. The solid circle represents the amount of X produced. (B) Solutions (100 μL) contained 130 μM H_2-neopterin-$(P)_3$ (chromatographically purified) and 0.055 M Tris-HCl that had been adjusted previously at 22 °C to the pH values shown. The reaction occurred at 100 °C for 10 min. The solid circles represent the combined amounts of X + X1, and the line is the concentration calculated for the nonprotonated form of tris(hydroxymethyl)aminomethane. The upper scale of pH is values measured at 22 °C, and the lower scale is values calculated for 100 °C assuming a change in pK of −0.031/deg.

Support for this hypothesis comes from the observations that (1) purified X gives rise to small amounts of X2 and X1 during storage at −20 °C, (2) as much as 50% of the purified X2 can be converted to X1 by storage at −20 °C for a week, and (3) neither X1 nor X2 have given rise to X either at −20 °C or at 25 °C. Because of these interrelationships and the observation that X1 can serve as a substrate for the enzymatic synthesis of sepiapterin, we believe that H_2-neopterin-$(P)_3$ is converted first to X and then probably either to X2 or to both X1 and X2; X1 is apparently the most stable form of the three.

The requirements for the nonenzymatic synthesis of X were examined (1) to optimize the conditions so that enough purified X, X1, and X2 could be produced for characterization and (2) to determine the mechanism of the nonenzymatic synthesis as an aid in understanding the enzymatic mechanism for the synthesis of X.

The rate of nonenzymatic X synthesis increased rapidly with temperature above 80 °C (Figure 3). Ionic strength had no effect on the reaction rate in the range tested (0.067–0.167 M NaCl) (data not shown).

The nonenzymatic synthesis of X demonstrated a marked pH optimum (Figure 4A). To understand why the reaction rate falls off at higher pH values, we considered five distinct possibilities. (1) The phosphate groups of H_2-neopterin-$(P)_3$ are hydrolyzed at the high pH. (2) The side chain of H_2-neopterin-$(P)_3$ is labile at high pH. Storage of H_2-neopterin-$(P)_3$ under alkaline conditions is known to give rise to xanthopterin. (3) Degradation of X could increase with increasing pH. (4) The ionization of the pterin moiety of H_2-neopterin-$(P)_3$ could decrease the reaction rate. (5) The presence of EDTA and NaCl caused another reaction to occur at the higher pH values.

These possibilities were examined as follows:

(1) Phosphate analysis revealed no significant loss of phosphate above pH 9.4 or below pH 7; a small amount of phosphate is released between pH 7 and 9.4. Although the synthesis of X may account for some of it, the amount is stoichiometrically 10-fold greater than could be accounted for by the synthesis of X alone. The amount still represents at the most 5% of the H_2-neopterin-$(P)_3$ present; since phosphate is not detected in reactions carried out at pH values greater than 9.4, it is concluded that loss of phosphate from H_2-neopterin-$(P)_3$ does not account for the decrease in X synthesis at higher pH.

(2) Analysis of side chain release using [3′-^{3}H]H_2-neopterin-$(P)_3$ revealed no significant loss of the side chain at any pH tested.

(3) Significant degradation of X (60 °C for 120 min) did not occur between pH 4 and 11.

(4) Since the pK_a values of various 7,8-dihydropterins fall between 10.0 and 11.1 (Blakely, 1969), H_2-neopterin-$(P)_3$ may also lose a proton in this pH range. The temperature dependence of this proton dissociation is not known, but it is known that Tris exhibits a decrease of its pK of 0.031 pH unit/deg as the temperature increases (Good et al., 1966). If the temperature dependence for the proton dissociation of H_2-neopterin-$(P)_3$ is similar to that of Tris, then the decrease in the rate of production of X may be correlated to a proton dissociation. However, if the temperature dependencies of H_2-neopterin-$(P)_3$ and Tris are quite dissimilar, this argument may not be valid.

(5) The H_2-neopterin-$(P)_3$ was separated from Tris, EDTA, NaCl, and cyclohydrolase by chromatography on DEAE-cellulose using a gradient of ammonium bicarbonate. The H_2-neopterin-$(P)_3$ was associated with a yellow peak on the leading edge of the peak of residual GTP as detected by A_{330} and fluorescence. The TLC assay demonstrated a strongly fluorescent substance intermediate in mobility to GDP and GTP. The pH dependence of the nonenzymatic synthesis of X was repeated by using tris(hydroxymethyl)aminomethane and chromatographically isolated H_2-neopterin-$(P)_3$ as the components of the aqueous solution. As seen in Figure 4B, a similar pH dependence was observed; the rate increased up to pH 9.5 (as measured at 22 °C) and thereafter decreased.

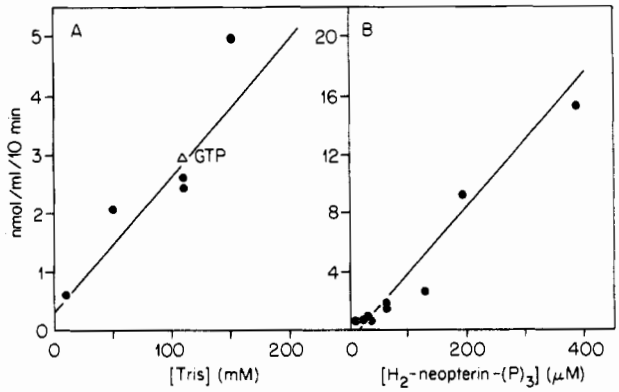

FIGURE 5: Kinetics of the nonenzymatic synthesis of X and X1. The reaction was performed at 100° for 10 min. (A) Solutions (100 μL) contained 130 μM H_2-neopterin-$(P)_3$ (chromatographically isolated) and various concentrations of Tris-HCl (pH 9.0). In one tube 0.15 mM GTP was present (Δ). (B) Solutions (100 μL) contained 0.11 M Tris (pH 9.0) and various concentrations of H_2-neopterin-$(P)_3$ (chromatographically isolated). In both experiments the amounts of X and X1 were added together; no X2 was detected. The lines were fitted to the experimental points by linear regression. The line for Tris is $y = 0.026x + 0.306$ with a correlation of 0.91; the line for H_2-neopterin-$(P)_3$ is $y = 0.042x - 0.689$ with a correlation of 0.98.

The amounts of X and X1 were combined for Figure 4B; the latter was usually 10%, but never more than 20%, of the former. No X2 was observed. The reaction is probably due to the attack on H_2-neopterin-$(P)_3$ by the amine since the points in Figure 4B represent the amount of X + X1 produced, and the line is the calculated amount of free amine. Up to pH 9.5 there is close correlation. As mentioned above the deprotonation of the pterin moiety could explain the decreased rate above pH 9.5.

Effect of Amine and H_2-neopterin-$(P)_3$ on the Rate of X Synthesis. When the chromatographically isolated H_2-neopterin-$(P)_3$ was used, the rate of X synthesis was shown to be first order with respect to tris(hydroxymethyl)aminomethane (Figure 5A) and also with respect to H_2-neopterin-$(P)_3$ (Figure 5B) when the pH was 9.0. When additional GTP was added to the reaction, there was no appreciable effect on the rate (Figure 5A). When the H_2-neopterin-$(P)_3$ was stored dry at 25 °C in the lyophilizing flask, three or four fluorescent components appeared on the thin-layer chromatogram; the rate of X synthesis did not differ appreciably when these components were absent or present.

The presence or absence of GTP cyclohydrolase, and the NaCl and EDTA associated with its reaction, had no effect on the analysis for X and X1. Since the elution positions of X and X1 in the experiments for Figures 4B and 5A,B were identical with the positions obtained in experiments similar to that in Figure 1, the chromatography is not altered by the presence of GTP cyclohydrolase or the other components in the sample.

Properties of X, X1, and X2. The ultraviolet spectra of X, X1, and X2 at pH 7.0 and 11.7 are shown in Figure 6. The long wavelength absorption maxima of all three compounds undergo a bathochromic shift with increasing pH, indicating that all three have a pK_a value between 7.0 and 11.7, as do most 6-alkylpterins.

Since enzymatically synthesized X incorporates label from [3′-^{3}H]H_2-neopterin-$(P)_3$ and since X and X1 can both serve as precursors for the synthesis of sepiapterin, it is clear that they retained the three-carbon side chain from H_2-neopterin-$(P)_3$. We assumed that at least two of the oxygen substituents of the side chain are retained and expected X, X1, and X2 to be sensitive to oxidation with $NaIO_4$. Accordingly

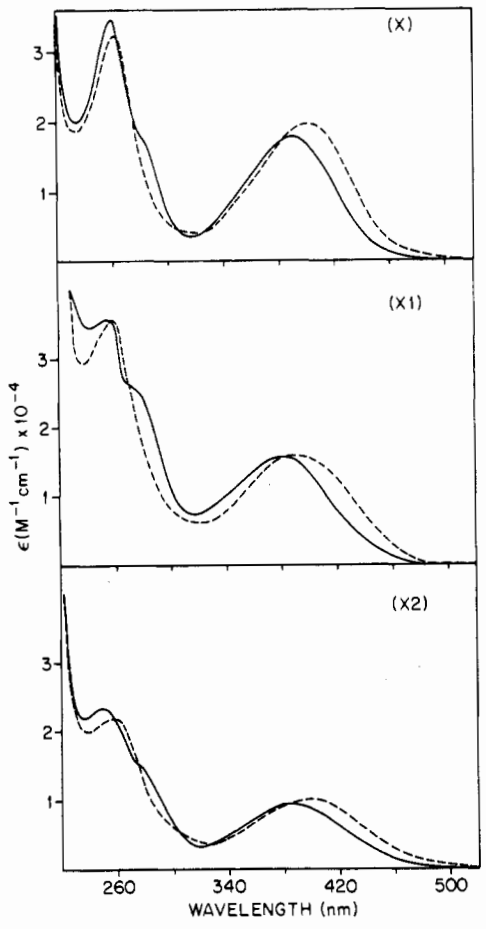

FIGURE 6: Ultraviolet absorption spectra of X, X1, and X2, at pH 7.0 and 11.7. Molar absorption coefficients were obtained by comparing A_{260} peaks of aliquots of purified compounds on HPLC to A_{260} peaks of the compounds generated by heating a solution of [U-^{14}C]H_2-neopterin-$(P)_3$ at 100 °C for 40 min. The nanomoles per unit peak area was calculated assuming that X, X1, and X2 were the same specific activity as the [U-^{14}C]H_2-neopterin-$(P)_3$ from which they were generated. The solid line is at pH 7.0 and the broken line at pH 11.7.

each was treated with this reagent and chromatographed on the C_{18} reversed-phase column. Only the X1 peak was lost, but X and X2 appeared in their usual elution position. Since X and X2 are apparently precursors of X1, we believe that all three contain potentially periodate-sensitive groups but that perhaps structural constraints prevent the oxidation of X and X2. Alternatively it is possible that periodate oxidation of X and X2 did not alter their chromatographic behavior; we demonstrated that periodate oxidation of sepiapterin did cause it to elute to a different position than sepiapterin and of course X1 was also altered. It seems most likely that X and X2 simply failed to react.

None of the compounds had their chromatographic properties on the C_{18} reversed-phase column altered by treatment with $NaBH_4$; this may indicate that no carbonyl groups are present in the side chain or that the reduced material is chromatographically indistinguishable from the normal material. This also may indicate that the periodate-sensitive group in X1 is due to vicinal hydroxyl groups. For a positive control sepiapterin was treated with $NaBH_4$, and it was completely removed from its typical position in the chromatogram.

Recently, iodine has been demonstrated to be a means of oxidizing the pyrazine ring of pterins (Fukushima & Nixon, 1979b). When X, X1, and X2 were oxidized by iodine, they appeared on the C_{18} chromatogram in the positions shown in Figure 1 as oxidized forms; it is from this evidence that the peaks that appeared after H_2-neopterin-(P_3) was boiled were

Table II: Chromatographic Behavior of X, X1, X2, and Some Common Pteridines[a]

compound	rel volume
sepiapterin	100
biopterin	50
pterin	46
X	168
X1	89
X2	214
xanthopterin	32
isoxanthopterin	48

[a] Each pteridine was chromatographed on a C_8 column at 35 °C with 12% methanol in water at a flow rate of 2 mL/min. Sepiapterin emerged at 11.2 mL. The relative positions are similar on the C_{18} column.

designated as oxidized X, X1, and X2 (Figure 1). These compounds are more fluorescent than the parent compounds, as would be expected from the oxidation of the pyrazine ring. Since the parent compounds are fluorescent, they are probably dihydropterins. When iodine oxidation products were reduced with $NaBH_4$, the parent compounds reappeared on the chromatograph; this again is evidence that the parent compounds (X, X1, and X2) are dihydropterins.

The absence of phosphate in X, X1, and X2 is apparent from their retention on the C_{18} reversed-phase column. Phosphoylated compounds, such as nucleotides and H_2-neopterin-$(P)_3$, are known to elute in ~1 column volumes (~3 mL). The elution positions of X, X1, and X2 are shown in Table II and compared to several common pteridines.

Discussion

The reaction of H_2-neopterin-$(P)_3$ with Tris to produce X, X1, and X2 must be viewed critically to establish the identity of X. Three criteria indicate that the X synthesized by chemical and by enzymatic procedures are identical: (a) the two products behave identically on high-performance liquid chromatography, (b) the absorption and fluorescent properties of the chromatographic peaks of the chemically and enzymatically produced X are the same, and (c) the two products are converted to sepiapterin by an enzyme preparation from *Drosophila*. Tanaka et al. (1981) reported a compound X that is produced from H_2-neopterin-$(P)_3$ by a purified enzyme from chicken kidney, and they suggested that it is 6-(1,2-dioxypropyl)-7,8-dihydropterin. There are two apparent discrepancies between the proposed structure of compound X of Tanaka et al. and the X that was characterized here: (a) X did not react with $NaIO_4$ and (b) X did not react with $NaBH_4$. Both would be expected from the proposed dicarbonyl structure. However, since Tanaka et al. did not report any attempt to measure a reaction with $NaIO_4$ or $NaBH_4$, it remains possible that X and compound X behave anomalously toward these reagents; Dyer (1956) described certain glycols that fail to react with $NaIO_4$. The most direct approach to resolve these possible discrepancies would be to compare compound X produced by the chicken kidney enzyme and the X produced chemically and by the *Drosophila* enzyme. It seems likely that the C_{18} reversed-phase column would detect any alteration caused by $NaIO_4$ and $NaBH_4$ since it readily resolves biopterin, sepiapterin, and oxidized sepiapterin.

The ultraviolet absorption spectra of X and the related compounds deserve comment. The long wavelength absorption maxima of most 6-alkyl-7,8-dihydropterins (e.g., 6-methyl-7,8-dihydropterin) exhibit a bathochromic shift with increasing pH as do many pteridines (Blakely, 1969). Few 6-alkyl-7,8-dihydropterins exhibit absorption maxima at wavelengths greater than 355 nm whereas X, X1, and X2 all have maxima

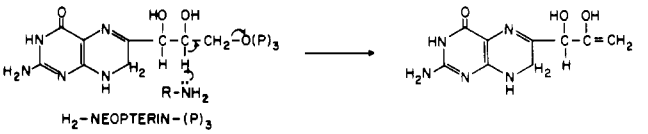

FIGURE 7: Postulated reaction mechanism for amine-catalyzed conversion of H_2-neopterin-$(P)_3$ to X.

at 380 nm or greater. Sepiapterin is closely related in that it has a bathochromic shift with increasing pH and an absorption maximum at 420 nm at pH 7 (Tanaka et al., 1981). These observations indicate that the three unknowns are closely related structurally and also that their properties are similar to those of sepiapterin.

In the three-carbon side chain of H_2-neopterin-$(P)_3$, each carbon is at the oxidation level of an alcohol, whereas in the 1,2-dioxypropyl side chain proposed for compound X (Tanaka et al., 1981), there is a net increase in the oxidation state. Tanaka et al. postulate that the chicken kidney enzyme has an electron acceptor that accomplishes the oxidation of the side chain. Since no oxidizing agent is added in the nonenzymatic synthesis of X, any increase in the oxidation of one H_2-neopterin-$(P)_3$ would need to be accompanied by a reduction of another H_2-neopterin. This possibility is ruled out by the fact that the nonenzymatic reaction is first order with respect to H_2-neopterin-$(P)_3$. We propose a second-order reaction in which the side chain of H_2-neopterin-$(P)_3$ undergoes a nucleophilic attack by the nonprotonated amine. Since ^3H is retained at the 3′-C of X, the site of attack is most likely at the 2′-C. This amine-catalyzed elimination reaction results in the release of the phosphates and the formation of a 1-hydroxyacetone moiety as shown in Figure 7. This is analogous to the enzymatic mechanism proposed by Krivi & Brown (1979). However, the failure of X to react with $NaIO_4$ or $NaBH_4$ raises the possibility that the final structure of X is not that shown nor is it that of Tanaka et al. (1981), as discussed above.

The pH optimum for the nonenzymatic reaction was measured under two conditions, one with Tris alone and the other with Tris, EDTA, and NaCl present. The temperature dependence of the pK of amines is well-known, and for Tris the pK is 8.1 at 20 °C but is 5.62 at 100 °C. EDTA is not known to shift its pK values in this manner so the net effect on pH at 100 °C will be different in the presence and absence of EDTA. The pH optimum with EDTA present is between 8.5 and 9 but in its absence is between 9 and 9.5. In both cases, pHs at still higher values resulted in lower rates of synthesis of X. In all cases, it should be recognized that the pH values at 100 °C are lower than those specified for 22 °C by 2.42 pH units.

Not only is X a precursor of sepiapterin but also we hypothesized that it is the precursor of *quench spot*, a drosopterin precursor (Dorsett & Jacobson, 1982). *Quench spot* retains all 11 carbons and appears to be a pyrimidodiazepine, a pyrimidine ring fused to the seven-membered diazepine (Jacobson & Dorsett, 1981; Wiederrecht et al., 1981). The identification of X opens the possibility of studying conditions to increase the yield of its formation and to seek conditions to stabilize it. The availability of X will facilitate studies on the mechanism of formation of sepiapterin and *quench spot* as well as leading to a further understanding of X1 and X2.

Acknowledgments

We thank Dr. John Yim for the initial observation of the formation of X at 100 °C and his continued interest in and discussions about this work. We are indebted to W. G. Hearl

for a generous supply of GTP cyclohydrolase I that was used in part of this study.

References

Blakeley, R. L. (1969) in *The Biochemistry of Folic Acid and Related Pteridines*, pp 58–76, North-Holland Publishing Co., Amsterdam.
Circular OR-10 (1956) Pabst Laboratories.
Dorsett, D., & Jacobson, K. B. (1982) *Biochemistry 21*, 1238–1243.
Dorsett, D., Yim, J. J., & Jacobson, K. B. (1979) *Biochemistry 12*, 2596–2600.
Dorsett, D., Yim, J. J., & Jacobson, K. B. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 39*, 1700.
Dyer, J. R. (1956) in *Methods of Biochemical Analysis* (Glick, D., Ed.) pp 111–153, Interscience, New York.
Fukushima, T., & Nixon, J. C. (1979a) in *Chemistry and Biology of Pteridines* (Kisliuk, R. L., & Brown, G. M., Eds.) pp 35–36, Elsevier/North-Holland, New York.
Fukushima, T., & Nixon, J. C. (1979b) in *Chemistry and Biology of Pteridines* (Kisliuk, R. L., & Brown, G. M., Eds.) pp 31–34, Elsevier/North-Holland, New York.
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., & Singh, R. M. M. (1966) *Biochemistry 5*, 467–477.
Jacobson, K. B., & Dorsett, D. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1547.
Krivi, G. G., & Brown, G. M. (1979) *Biochem. Genet. 17*, 371–390.
Lewis, E. B. (1960) *Drosophila Inf. Serv. 34*, 117.
Matsubara, M., Katoh, S., Akino, M., & Kaufman, S. (1966) *Biochim. Biophys. Acta 122*, 202–212.
Neal, M. W., & Florini, J. R. (1973) *Anal. Biochem. 55*, 328–330.
Sumner, J. B. (1944) *Science (Washington, D.C.) 100*, 413–414.
Tanaka, K., Akino, M., Hagi, Y., Doi, M., & Shiota, T. (1981) *J. Biol. Chem. 256*, 2963–2972.
Tsusue, M., & Akino, M. (1965) *Dobutsugaku Zasshi 74*, 91–94.
Wiederrecht, G. J., Paton, D. R., & Brown, G. M. (1981) *J. Biol. Chem. 256*, 10399–10402.
Wilson, T. G., & Jacobson, K. B. (1977) *Biochem. Genet. 15*, 307–319.
Yim, J. J., & Brown, G. M. (1976) *J. Biol. Chem. 251*, 5087–5094.
Yim, J. J., Grell, E. H., & Jacobson, K. B. (1977) *Science (Washington, D.C.) 198*, 1168–1170.
Yim, J. J., Crummett, D., & Jacobson, K. B. (1981) *Insect Biochem. 11*, 363–370.

Reactivation of D-β-Hydroxybutyrate Dehydrogenase with Short-Chain Lecithins: Stoichiometry and Kinetic Mechanism†

Jorge D. Cortese,‡ Juan C. Vidal,§ Perry Churchill,‖ J. Oliver McIntyre, and Sidney Fleischer*

ABSTRACT: D-β-Hydroxybutyrate dehydrogenase (BDH), purified as soluble, lipid-free apoenzyme (inactive) from either beef heart or rat liver mitochondria, can be reactivated by short-chain lecithins in the monomeric state. The enzyme was reactivated with dihexanoyl- [PC(6:0)], diheptanoyl- [PC(7:0)], and dioctanoyllecithins [PC(8:0)]. The titration curves of enzyme activity as a function of the phospholipid concentration are consistent with a model in which the enzyme contains two identical, noninteracting lecithin binding sites. The simultaneous occupation of these sites (via an equilibrium random mechanism) is required to activate the apoenzyme. Similar results were obtained with both rat liver and beef heart apoenzymes. The maximal velocities obtained with the different lecithins were similar [110–140 μmol of NAD^+ reduced min^{-1} (mg of protein)$^{-1}$]. The K_L values (the apparent dissociation constants of the lecithin-site complexes) were 1.2×10^{-4} M [PC(8:0)], 1.5×10^{-3} M [PC(7:0)], and 4.5×10^{-3} M [PC(6:0)] at 37 °C. This was confirmed by using phospholipase A_2 to compete with the dehydrogenase for the lecithin monomers. Comparison of the $\Delta G°$ values for complex formation with the different lecithins shows an average contribution of approximately 2.4 kJ/mol ($0.9RT$) per CH_2 group. The interaction of the apolar moiety of lecithin with the protein seems to be essential for effective binding of phosphatidylcholine to apoBDH. The $\Delta G°$ values, when combined with the estimated $\Delta H°$ values, suggest that the binding of lecithin to the apoenzyme is ~60% enthalpy and ~40% entropy driven.

Biological membranes are highly organized structures that consist mainly of phospholipid and proteins. D-β-Hydroxybutyrate dehydrogenase [D(−)-3-hydroxybutyrate:NAD^+ oxidoreductase, EC 1.1.1.30; henceforth BDH[1]] is one of the best studied phospholipid-requiring enzymes (Fleischer et al., 1974). It is tightly bound to the mitochondrial inner membrane

† From the Departamento de Química Biológica (J.D.C. and J.C.V.), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina, and the Department of Molecular Biology (P.C., J.O.M., and S.F.), Vanderbilt University, Nashville, Tennessee 37235. *Received October 27, 1981; revised manuscript received April 19, 1982.* Supported in part by a grant from Fundación "Lucio Cherny" and by National Institutes of Health Grant AM 14632.

‡ Research Fellow of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.
§ Career Investigator of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.
‖ Recipient of a National Institutes of Health fellowship to P. Churchill (IFAM063272).

[1] Abbreviations: BDH, D-β-hydroxybutyrate dehydrogenase; apoBDH, D-β-hydroxybutyrate dehydrogenase apoenzyme; PC, phosphatidylcholine; MPL, a mixture of phosphatidylcholine, phosphatidylethanolamine, and diphosphatidylglycerol (molar ratio 1.0:0.8:0.2) isolated from rat liver mitochondria; PC(18:1), PC(8:0), PC(7:0), and PC(6:0), 1,2-dioleoyl-, 1,2-dioctanoyl-, 1,2-diheptanoyl-, and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine, respectively; D-PC(7:0), 2,3-diheptanoyl-*sn*-glycero-1-phosphocholine; cmc, critical micellar concentration; DEAE, diethylaminoethyl; CM, carboxymethyl.

0006-2960/82/0421-3899$01.25/0